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Effects of titratable acidity and organic acids on enamel erosion *in vitro*

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지도교수 진 보 형

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Abstract

Effects of titratable acidity and organic acids on enamel erosion *in vitro*

Kim Eun-Jeong, BSDH, RDH

(Directed by Associate Prof. Jin Bo-Hyoung, DDS, MSD, PhD)

Department of Preventive and Social Dentistry,

The Graduate School, Seoul National University

The aim of the present study was to determine the erosive potential difference among four naturally acidic fruit nectars (mandarin, orange, lemon, grapefruit) within the same range of titratable acidity and the influence of the components of organic acids on dental erosion.

Diluted fruit nectars (mandarin 1:1.1, orange 1:1.7, lemon 1:15, grapefruit 1:20) with the same range of titratable acidity (7.9 mL) were used. Specimens were randomly allocated to each group and were placed in a conical tube with 50 mL of test solutions for 1 h. Before and after procedure, enamel erosion was measured by micro-hardness test, CLSM and SEM ($p > 0.05$). The separation of acids was carried out using a high performance liquid chromatography to analysis composition of each test solution.

Enamel erosion occurred with all test groups and showed similar decrease in VHN (no statistically differences were founded in the enamel surface hardness after erosion). The surface roughness changes were similar in the diluted orange, diluted lemon, and diluted grapefruit juices. The changes in the diluted mandarin nectar were different from those in the other experimental groups. The specimens of each group showed different patterns of enamel surface by SEM images. The citric and malic acids were the major organic acids in all test fruits. The lemon and orange groups had the highest malic acid concentrations; the mandarin group had the lowest malic acid concentration.

The titratable acidity, citric and malic acids of fruits could be crucial factors in enamel erosion; therefore, fruit-based drinks should be regarded as potentially erosive.

Keywords : Confocal laser scanning microscopy, liquid chromatography, Microhardness, Organic acid, Titratable acidity, Tooth Erosion

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1. Introduction

In recent studies, dental erosion has become increasingly recognized as a major factor of tooth loss among all age groups.[1] Studies of 12-year-old children in the UK midlands reported that approximately 60% of these children had signs of dental erosion.[2] Two to five year olds showed erosion on deciduous teeth in 6-50% of the subjects, 5-9 year olds already had erosive lesions on permanent teeth in 14% of the cases, and 11-100% of 9-17 year olds showed signs of erosion in Sweden.[3] Similar results were reported for 5-year-old Irish school children, of whom 47% exhibited some erosion.[4]

Dental erosion is defined as the loss of dental hard structure due to chemical dissolution without bacteria in the mouth.[5] The complex and multi-factorial etiology of dental erosion is attributed to a wide range of extrinsic and intrinsic factors.[6] The extrinsic causes of dental erosion include environmental, dietary, medication and lifestyle factors. In Western societies, diet is thought to be the main extrinsic factor in the etiology of dental erosion and has received the most attention in the dental literature.[7]

Dietary erosion is attributed to the excessive intake of acidic beverages, acidic fruits, and fruit juices.[8] The most frequently consumed erosive acids are phosphoric and fruit acids, predominantly citric and malic acid.[9] Citric and malic acids are the major organic hydroxyl acids found in fruit and fruit juices. The erosive activity of citric, malic, phosphoric, and other acids has been investigated and demonstrated in many in vitro, in situ and in vivo studies.[9-12]

Traditionally, it has been believed that pH is an accurate indicator of the erosive potential of an acid;[13] however, the pH is an indicator only of the number of hydrogen ions that are present in solution and not of the presence of undissociated acid, whereas titratable acidity is a measure of the total acid content that allows beverages to resist pH change. Thus, measuring titratable acidity could be a more useful means of predicting erosive potential.[14] Titratable acidity is widely accepted as a crucial factor in measuring dental erosion.[15-19]

Many young children drink large quantities of dilutable fruit drinks.[20] Beverage trends in the United States include an increased consumption of sodas, 100% juices and juice drinks.[18] Chinese adults living in Hong Kong frequently consume fruit and lemon tea/water.[21] This phenomenon is also observed in Korea. Free trade agreements with many countries, such as the USA, and internationalization have increased fruit importation and consumption, among other dietary changes. The increase in fruit juice consumption may be reflected by an increase in cases of erosion.[10] Erosion is a gradual process; these dietary changes have occurred fairly quickly, and the full extent of the erosive effects of acidic beverages is not yet clear.[18]

Despite the increase in the consumption of fruits and fruit juices, dental erosion has received little attention. There is limited information available on many variables. Most studies have measured only pH or titratable acidity. However, few studies which set same titratable acidity have been defined. We hypothesized that if we held the same titratable acidity to acidic fruit nectar, the potential dental erosion factors could be more precisely examined and analyzed for their organic acid compositions. The aim of the present study was, therefore, to confirm the erosive potential difference among four naturally acidic fruit nectars within the same range of titratable acidity. Additionally, the influence of the components of the organic acids on dental erosion was studied.

2. Materials and methods

2.1. Selection of products and preparation of the test solutions

Four types of acidic fruit were selected in this study: the orange, which is very common; the lemon, which is the sourest fruit; the grapefruit, which is becoming increasingly common; and the mandarin, which is popular fruit in Korea.

An electric juicer (Hurom, Kimhae, Korea) was used to prepare fruit nectars. Each of fruit nectar was centrifuged for 30 min at 8,000 rpm using a high-speed centrifuge (Thermo Electron Laboratory Equipment, RC-6 plus, Waltham, MA, USA). After centrifugation, the supernatant was collected, and the diluted fruit nectars were adjusted to the same titratable acidity level. The titratable acidity was measured using a pH electrode (Orion ROSS™, 8102BNUWP, Beverly, MA, USA) connected to a pH meter (OrionStar™, Beverly, MA, USA). The fruit nectar dilutions were prepared with distilled water as the diluent. Twenty milliliters of each of diluted fruit nectar was placed in a glass beaker at 37 °C and was titrated by adding 7.9 mL of 0.1 M sodium hydroxide solution and measuring the pH until pH 5.5 was reached.

The pH of each experimental group, in which all samples had the same titratable acidity, was measured by Hunter's method.[16] Between measurements, the pH electrode was rinsed with distilled water. This process was measured three times.

2.2. Specimen preparation

For this study, extracted bovine incisors without noticeable cracks, disinfection or any lesions under quantitative light-induced fluorescence (QLF, QLF Pro®, Inspektor Research System BV, Amsterdam, Netherlands) were selected. Small holes with a diameter of 5mm were drilled at the top of the enamel surface. The samples were placed in molds measuring 1.2 x 1.0 x 0.8 cm with the labial surface embedded in acrylic resin. The specimens were polished and flattened with silicon carbidepaper (600-2,000 grid) under cool water.

All prepared specimens were stored in a constant relative humidity of 100% prior to use.

2.3. Experimental procedure

A total of 48 specimens were randomly allocated to each group of diluted fruit nectar and were placed in a conical tube with 50 mL of the test solution. The conical tubes were then placed in a thermostatically controlled agitator at 37 °C with a speed of 150 rpm and were stirred for 1 h. After exposure, the specimens were removed from the experimental solution and rinsed with distilled water.

2.4. Measurement of microhardness and surface roughness

The enamel microhardness of the specimens was determined using a Vickers microhardness tester (Shimadzu, HMV-2, Kyoto, Japan). Three indentations were measured for 10 s using a diamond at 9.81 N with 40 X magnification lenses. The average microhardness was calculated.

The surface roughness of the specimens was measured using confocal laser scanning microscopy (CLSM). The enamel surface roughness images were captured as a hundred serial images with 5 μm spacing on the Z-axis as the specimen's center. The microhardness and surface roughness images were analyzed in triplicate both before and after the experimental procedure.

2.5. Scanning electron microscopy

In order to observe the enamel surface, specimens were sputter-coated with platinum and observed in a scanning electron microscope (S-4700, HITACHI, Tokyo, Japan). The surfaces were photographed at a magnification of ×5000 and 15 kV accelerating voltage.

2.6. Liquid chromatographic analysis

The separation of acids was measured using a high performance liquid chromatograph (Dionex, Ultimate 3000, CA, USA) equipped with a 250 mm x 4.6 mm x 5 m C-18 column (Innopia, Inno column, Gyeonggi-do, Korea). The sample thermostat was maintained at 25 °C. The injected sample volume was 10 μL, and the mobile phase was a gradient of A (20 mM KH_2PO_4) and B (Acetonitrile): 0-15 min 100% A, 15-20 min 100% B, 20-25 min 100% B, 25-26 min 100% A, 26-30 min 100% A. The flow rate was 0.5 mL/min, and the detector was performed at UV 215 nm.

2.7. Statistical analysis

Data obtained for the surface roughness (Ra) and microhardness were statistically analyzed by both a one-way analysis of variance with four experimental groups and a post hoc Tukey's honestly significant differences (HSD) test using the studentized range. The threshold for statistical significance was set at $p < 0.05$. The SPSS (Statistical Packages for Social Science, Ver. 19.0, Chicago, IL, USA) statistical program was used for statistical analyses.

3. Results

3.1. Initial pH of each group of diluted fruit nectars adjusted to the same titratable acidity

Among the four groups of each diluted fruit juice, the diluted lemon nectar (1:15) had the lowest pH (2.74), the diluted mandarin nectar (1:1.1) had a pH of 3.58, the diluted orange nectar (1:1.7) had a pH of 3.82, and the diluted grapefruit nectar (1:20) had the highest pH (4.02).

3.2. The enamel microhardness change after the experimental procedure

The baseline microhardness had a range of 308.1–314.8 VHN. Table 1 shows the mean enamel surface hardness after exposure to four different diluted fruit nectar groups. These groups of diluted fruit nectar led to similar decreases in VHN after treatment: diluted mandarin nectar (75.9 △VHN), diluted lemon nectar (89.1 △VHN), diluted grapefruit nectar (91.7 △VHN), and diluted orange nectar (92.5 △VHN). No statistically significant differences were found in the enamel surface hardness after erosion.

3.3. The enamel surface roughness change as determined by CLSM imaging after the experimental procedure

The exposure of the enamel surface to the four different groups of diluted fruit nectar for 1 hour resulted in changes in the appearance of the surface for each of the tested solutions. The changes were similar in the diluted orange, diluted lemon, and diluted grapefruit juices. The changes in the diluted mandarin nectar group were different from those in the other experimental groups (Table 2, Fig. 1).

Table 1. Comparison of the surface microhardness of different groups before treatment and after erosion by diluted fruit nectars

Diluted fruit nectars	N	VHN*		ΔVHN**
		Before treatment	After treatment	
Diluted Fruit nectars				
Mandarin : water (1:1.1)	12	314.8± 7.8	238.9±15.2	75.9±14.7[a]
Orange : water (1:1.7)	12	308.1±14.7	215.6±15.7	92.5±15.7[a]
Lemon : water (1:15)	12	313.2±12.7	224.1±19.2	89.1±19.9[a]
Grapefruit : water (1:20)	12	314.6±10.9	222.8±8.8	91.7±12.8[a]

* Values are mean ± SD.
**ΔVHN = After treatment VHN – Before treatment VHN.
[a] The different characters indicate statistically significant differences between groups by Tukey's HSD post hoc test at $p > 0.05$.

Table 2. Surface roughness (Ra) measurements by CLSM

Diluted fruit nectars	N	Surface Roughness (Ra)*		ΔRa**
		Before treatment	After treatment	
Diluted Fruit nectars				
Mandarin : water (1:1.1)	12	0.69±0.12	0.74±0.08	0.05±0.08[b]
Orange : water (1:1.7)	12	0.70±0.14	1.05±0.17	0.35±0.22[a]
Lemon : water (1: 15)	12	0.60±0.18	0.94±0.13	0.34±0.27[a]
Grapefruit : water (1: 20)	12	0.60±0.11	0.75±0.07	0.15±0.11[a]

* Values are mean ± SD0
** ΔRa = After treatment Ra – Before treatment Ra.
a,b The different characters indicate statistically significant differences between groups by Tukey's HSD post hoc test at p>0.05.

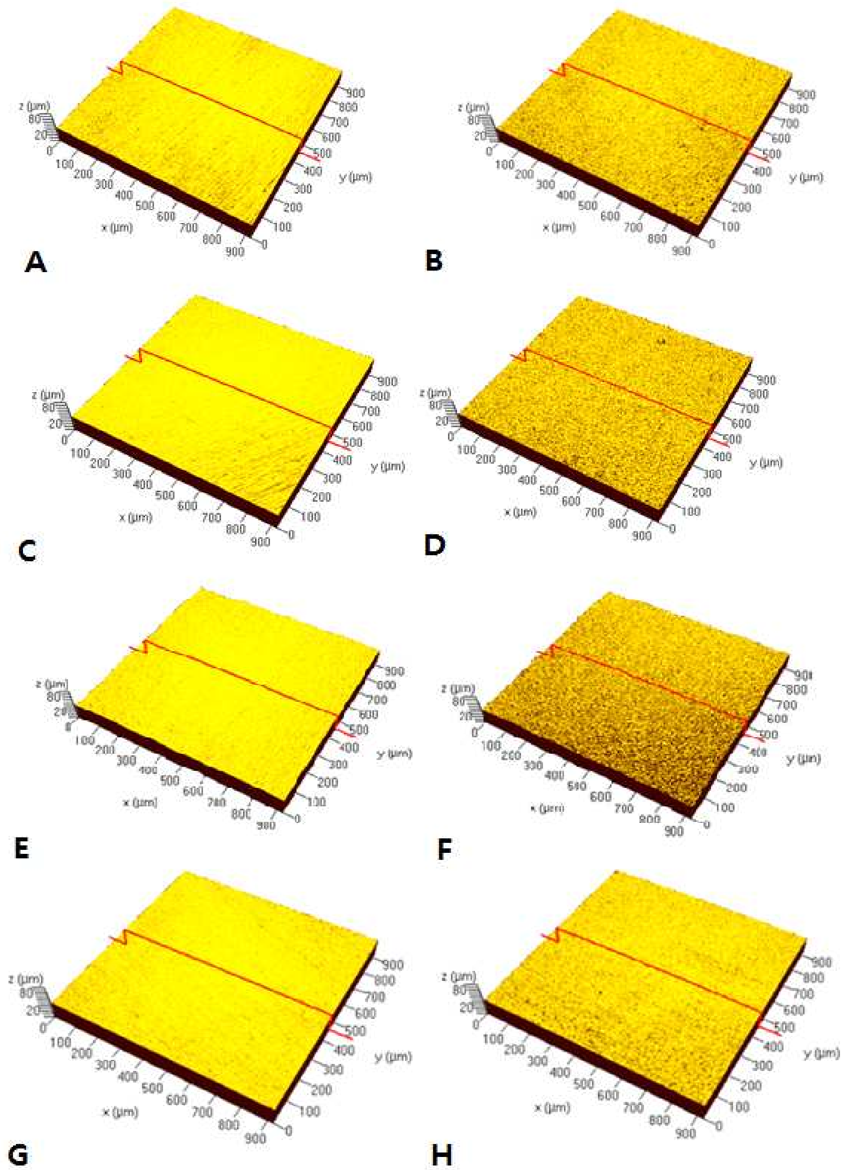

Fig. 1. CLSM images before (A) and after (B) treatment with diluted Mandarin nectars, before (A) and after (B) treatment with diluted Orange nectars, before (A) and after (B) treatment with diluted Lemon nectars, before (A) and after (B) treatment with diluted Grapefruit nectars.

3.4. Scanning electron microscopic observations

The SEM micrographs of enamel immersed in the each test solution were presented in Figure 3-2. The SEM images of specimens of the diluted lemon nectar was great demineralization, and dentinal tubules partially opened. The SEM images of specimens of diluted orange nectar showed irregular surface, traces of opened dentinal tubules. The SEM images of specimens of diluted grapefruit nectar showed different results to those lemon nectar and orange nectar, it had some patterns of demineralization of the enamel surface. And the SEM images of specimens of diluted mandarin nectar were also differed from that of other groups (Fig. 2).

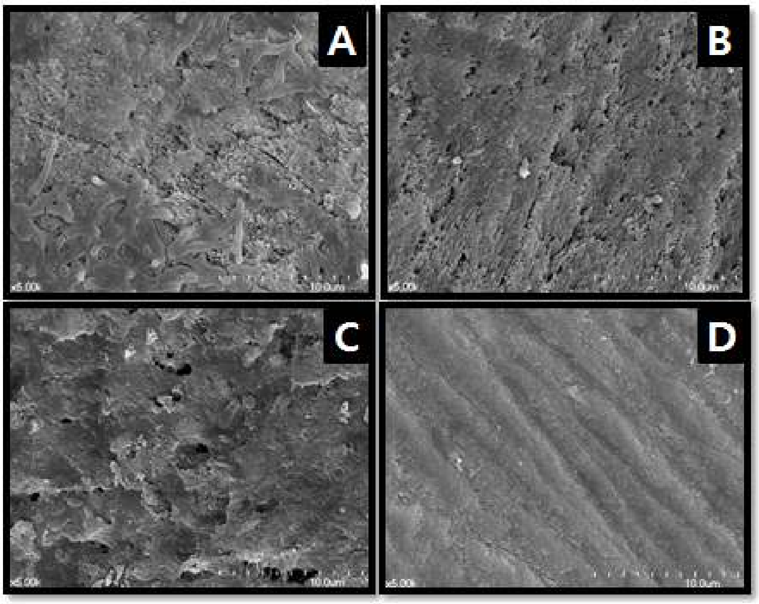

Fig. 2. Scanning electron micrograph of the specimens which had been placed in each test solution for 1 hours. ×5000. A = diluted mandarin nectar group. B = diluted orange nectar group. C = diluted lemon nectar group. D = diluted grapefruit nectar group.

3.5. Liquid chromatographic determination

Different concentrations of organic acid were tested as mobile phases to obtain the optimal response and separation of the organic acids. The citric and malic acids were the major organic acids in all test fruits. The lemon and orange groups had the highest malic acid concentrations; the mandarin group had the lowest malic acid concentration (Fig. 3).

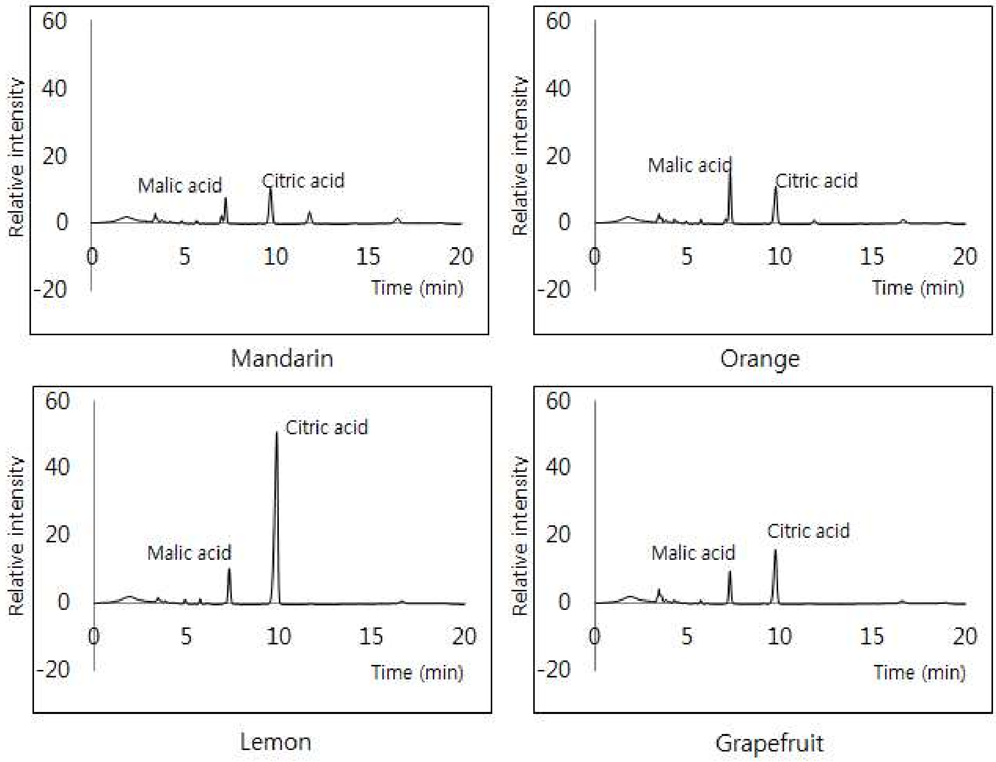

Fig. 3. Analysis of the liquid chromatograms obtained for the mandarin, lemon, orange and grapefruit nectar.

4. Discussion

The prevalence of dental erosion has risen over the last several decades[1] and has been the focus of dental research.[22] The most common extrinsic factor of dental erosion is the dietary factor; dietary erosion was ascribed to an excessive intake of acidic beverages, acidic fruit, and fruit juices.[8] The aim of the current study was to determine how diluted fruit nectar with the same titratable acidity affects the tooth structures and to analyze the influence of different organic acids on dental erosion.

In general, enamel eroded when the pH was below 4.5. The current study showed that enamel erosion occurred for all of the diluted fruit nectar groups. The results indicated that all the test solutions were highly acidic, even after they were diluted. These data are consistent with the results from a recent report by Cairns et al.[19], who found that a diluted test solution was highly acidic.

In this study, four types of fruit nectars were adjusted to the same titratable acidity. The enamel erosion lesion depths, measured using a microhardness test after *in vitro* exposure to the test solutions, were numerically similar; no significant difference was observed in the enamel hardness after erosion. It must be remembered that there are no other factors on dental erosion except titratable acidity. These findings differ from the results of Johansson et al.[23] However, titratable acidity has been demonstrated to be a better guide to the erosive potential of drinks, because it measures the actual quantity of H^+ ions available to interact with the tooth surface.[7] It has been generally demonstrated that titratable acidity is a better indicator of erosive potential than pH alone.[24,25] The results of the present study correspond well with those of an earlier study that reported that enamel erosion increases with the increased consumption of high acid.[26,27] This conclusion was also reached in the Cairns et al.[19] study. These previous studies had different experimental conditions than the present study, but all of the studies found that titratable acidity plays a crucial role in enamel erosion.

The effects of immersion of the enamel in these experimental solutions in vitro would be expected to be dependent on the titratable acidity of the solution. Nevertheless, the CLSM images of the surface roughness of the enamel after exposure to the four groups of diluted fruit nectar show little different results compared with microhardness test. The value of the surface roughness (Ra) after immersion in the diluted orange nectar, diluted lemon nectar and diluted grapefruit were similar. However, the diluted mandarin nectar was different from the other nectars. The reason for this disparity is not clear, but may be related to the types of organic acids and mineral ion concentrations in the fruit nectars. Chemical dissolution of teeth occurs by the hydrogen ion derived from the acids.[28] It can attack the tooth mineral crystals and directly dissolve them by combining with either the carbonate ion or the phosphate ion and releasing all of the ions from that region of the crystal surface, leading to direct surface etching. Acids such as citric acid have double actions and are highly damaging to the tooth surface.[29] In this study, orange, lemon and grapefruit nectar, which have high concentrations of citric and malic acids, were more erosive than mandarin nectar, which has low concentrations of citric and malic acids than others and shows different result in surface roughness (Ra). Grobler et al.[30] showed that the acid type and concentration are important in determining the amount of damage, even when the pH values are similar. Similar to previous studies, this phenomenon may assume that each organic acid has its own dissolving characteristics.

The SEM analysis presented a progressive destruction of the enamel ultrastructure, mostly specimens immersed in diluted lemon and orange nectar. The specimens exposed to the diluted orange nectar showed irregular demineralization of the enamel surface, these data are consistent with the results from a recent report by Torres et al.[31], who found that a soy-based orange juice showed irregular surface after 60-day immersion. In this study, the specimens of each group showed different patterns of enamel surface, this results may assume that the type and concentration of acid may influence the pattern of enamel surface.

The present study analyzed organic acids independently. Citric and malic acid were the major organic acids in the test group. The results of this study coincide well with those of an earlier study, which reported that citric acid represented the highest proportion, followed by malic acid in orange and lemon juice.[32] Citric acid is a complex organic acid with three acid dissociation constants; it has a particularly high erosive potential as a result of both its acidic nature and chelating properties, even when diluted. Concentrates with high levels of citric acid will retain a significant erosive potential.[16] Furthermore, malic acid was found in low concentrations in the mandarin groups and in high concentrations in orange and lemon groups. This result of the present study is quite similar to that of a previous study, which showed that malic acid was more erosive than citric acid at pH 2.8 and 3.3 and with a 0.6 g/100 mL acid concentration, and malic and citric acid were not different in their erosivity at pH 3.8 and a 0.35 g/100 mL acid concentration.[33] This phenomenon may assume that malic acid may also affect the enamel. From this results, there will be need to discover the effect of erosive potential about how malic acid affect to enamel surface compare with citric acid for further study.

This study has a limitation stemming from its in vitro study. It was impossible to recreate an oral environment perfectly. We only experimented with citrus fruit nectars; therefore, we could not consider the difference among many types of fruits.

In summary, these results suggest that titratable acidity and, more specifically, citric and malic acid in fruits could be a major factor in enamel erosion. Further studies should investigate what titratable acidity levels can help minimizes tooth erosion as well as the minimal concentration of acid needed to cause tooth erosion.

5. Conclusions

The purpose of the present study is to confirm the erosive potential difference among four naturally acidic fruit nectars within the same range of titratable acidity. Additionally, the influence of the components of the organic acids on dental erosion was studied.

1. The enamel erosion lesion depths, measured using a microhardness test after *in vitro* exposure to the test solutions, were numerically similar ($p > 0.05$).
2. The enamel surface roughness changes were similar in the diluted orange, diluted lemon, and diluted grapefruit juices.
3. The specimens of each group showed different patterns of enamel surface.
4. The citric and malic acids were the major organic acids in all test fruits.
5. The lemon and orange groups had the highest malic acid concentrations; the mandarin group had the lowest malic acid concentration.

The titratable acidity and citric and malic acids of fruits could be crucial factors in enamel erosion; therefore, fruit-based drinks should be regarded as potentially erosive. Consumers should be aware of this potential danger, and public policies should suggest preventive advice on the consumption of such beverages.

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국문초록

과즙 내 적정산도와 유기산 종류에 따른 치아부식도

서울대학교 치의학대학원 치의과학과 예방치학전공
(지도교수 : 진 보 형)

김 은 정

치아 부식증은 구강 내 세균과는 상관없이 화학적 작용에 의해 치아 경조직이 손실되는 것이다. 치아 부식증의 원인은 내인성 요인과 외인성 요인으로 나눌 수 있는데, 외인성 요인으로는 산성음료와 산성음식의 섭취, 생활습관, 환경, 산성 약물 등이 있다. 최근에는 외인성 요인 중 섭취하는 식품에 의하여 발생하는 치아 부식이 가장 주요 요인으로 보고되고 있다. 이에 본 연구는 4종류의 산성 과일(귤, 오렌지, 레몬, 자몽)의 적정산도를 동일하게 맞추어 법랑질 표면경도와 표면 거칠기의 변화로 치아 부식 정도의 차이를 알아보고, 과즙 내 유기산 분석을 통하여 치아표면의 부식의 영향을 평가하고자 하였다.

본 연구에서 사용된 4종류의 산성과일 즙을 적정산도가 7.9mL가 되도록

증류수를 첨가하여 희석시켰다. 각 군당 12개의 시편을 각각 50mL의 conical tube에 넣어 37°C, 150rpm으로 1시간 동안 부식시킨 후 증류수로 세척하였다. 각 시편은 부식 전, 후의 표면 경도와 거칠기를 3회 반복 측정 후 평균값을 기록하였다. 각 과일즙의 유기산 분석은 액체크로마토그래피(HPLC)를 사용하여 분석하였다.
4종류의 과일즙을 대상으로 적정산도 및 유기산과 치아부식증의 영향을 조사한 결과, 다음과 같은 결론을 얻었다.

1. 적정산도를 동일하게 맞춘 모든 실험 군에서 표면경도 감소의 차이가 비슷한 것으로 나타났다 ($p > 0.05$).
2. 희석 오렌지즙, 희석 레몬즙, 희석 자몽즙에서 표면거칠기 감소의 차이가 비슷한 것으로 나타났다.
3. 희석 오렌지즙, 희석 레몬즙, 희석 자몽즙, 희석 귤즙에서 각각 다른 패턴의 법랑질 표면 부식 양상을 보였다.
3. 구연산과 말산은 모든 실험 과일 군에서 주요산으로 나타났다.
4. 레몬즙과 오렌지 즙 군에서 말산의 함유량이 다른 군보다 높았으며, 귤 즙 군에서 가장 낮은 것으로 나타났다.

주요어 : 치아부식, 적정산도, 유기산, 액체크로마토그래피, 표면 경도, 표면 거칠기
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